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EVALUATION OF DIMETHYL METHYLPHOSPHONATE AND EXO-TETRAHYDRODI-(CYCLOPENTADIENE) IN A BATTERY OF *IN VITRO* SHORT-TERM ASSAYS

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Dimethyl methylphosphonate (DMMP) and exo-tetrahydrodi-(cyclcpentadiene) (JP-10), two compounds of interest to the United States Air Force, were evaluated in a battery of five in vitro short-term assays to assess their potential biological activity. The assays conducted include the Ames Salmonella/mammalian microsomal mutagenicity assay, the CHO/HGPRT gene mutation assay, the CHO/sister chromatid exchange assay, the CHO/chromosome aberrations assay, and the BALB/c-3T3 neoplastic transformation assay.

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In the presence of an S-9 fraction, DMMP produced no mutagenic response in the Ames Salmonella mutagenicity assays. Results on JP-10 were inconclusive.

SUMMARY

Dimethyl methylphosphonate (DMMP) and exo-tetrahydrodi(cyclopentadiene) (JP-10), two compounds of interest to the United States Air Force, were evaluated in a battery of five in vitro short-term assays to assess their potential biological activity. The assays conducted include the Ames Salmonella/mammalian microsomal mutagenicity assay, the CHO/HGPRT gene mutation assay, the CHO/sister chromatid exchange assay, the CHO/chromosome aberrations assay, and the BALB/c-3T3 neoplastic transformation assay.

In the absence of an exogenous metabolic activation system, DMMP exhibited a low clastogenic activity in the CHO/chromosome aberrations assay and was negative in the remaining four assays. Compound JP-10 also had a marginal clastogenic effect in the CHO/chromosome aberrations assay and produced a negative (or inconclusive) response in the other assays conducted.

In the presence of an S-9 fraction, DMMP produced no mutagenic response in the Ames Salmonella mutagenicity assays. Results on JP-10 were inconclusive.

PREFACE

This is the final report of work performed by Arthur D. Little, Inc., for the Air Force under Work Order #2, Contract F33615-81-D-0508, Work Unit 63020155, "Mutagenic, Teratogenic, and Carcinogenic Potential of Air Force Chemicals." This report describes accomplishments from July 15, 1982, to December 15, 1982. Andrew Sivak, Ph.D., was Program Manager for the program. Alice Tu, Ph.D., was Task Manager for this Work Order. Key personnel involved with this project included: Mildred Broome, Ph.D.; Charlotte Dougherty, B.A.; Wendy Coombes, B.S.; and Stacie Pallotta, B.S. Marilyn George, Biochemical Toxicology Branch, Toxic Hazards Division, Aerospace Medical Research Laboratory, was technical monitor for the Air Force.

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LIST OF ABBREVIATIONS

DMMP	dimethyl methylphosphonate
JP-10	exo-tetrahydrodi-(cyclopentadiene)
CHO	Chinese hamster ovary
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
SCE	sister chromatid exchange
CA	chromosome aberrations
S-9	9000 xg supernatnant
EMS	ethylmethanesulfonate
MCA	3-methylcholanthrene
FCS	fetal calf serum
FCM	macromolecular (dialyzed) fraction of fetal calf serum
EDTA	disodium ethylenediamine-tetraacetate
PBS	phosphate-buffered saline
DMSO	dimethylsulfoxide
BrdU	bromodeoxyuridine
KCl	potassium chloride
IITRI	Illinois Institute of Technology Research Institute

INTRODUCTION

The directed effort in "short-term" bioassay development over the last decade arose from a need for rapid, sensitive and reliable means of assessing the potential health hazards of existing and new environmental chemicals. The usefulness of *in vitro* tests in screening for hazardous chemicals was enhanced by the initial report of Ames *et al.* (1973), who reported that there was a good correlation between carcinogenic activity of chemicals and the induction of a mutagenic response in a microbial mutagenicity assay.

Since then, a wide variety of *in vitro* test systems have been reported. In 1979, Hollstein *et al.* estimated that over 130 assays were available. These various assay systems can be broadly divided into three categories, namely (1) those which detect mutagenic or chromosomal changes in microorganisms or mammalian cells, (2) those in which there is an induction of morphological transformation in mammalian cells in culture, and (3) those in which interactions between the chemical and target macromolecule such as DNA can be assessed. While no single assay has been found to correlate perfectly with *in vivo* carcinogenicity test results, it has been generally recognized that a tier or battery of these short-term assays will provide a reasonable early indication of this potential biological activity of environmental chemicals. In addition, positive findings in one or more of the genetic assays indicate a concern for potential hazard to the human gene pool.

Arthur D. Little, Inc., at the request of the United States Air Force, evaluated two compounds, dimethyl methylphosphonate and exo-tetrahydrodi-(cyclopentadiene), in a battery of *in vitro* short-term assays. The assays conducted were the Salmonella/mammalian microsomal mutagenicity assay (Ames assay), a mammalian specific locus gene mutation assay (CHO/HGPRT assay), two cytogenetic assays measuring sister chromatid exchange (CHO/SCE assay) and chromosome aberrations (CHO/CA assay), and a mammalian neoplastic transformation assay (BALB/c-3T3 assay). These assays are all well validated systems and are representative of varied endpoints designed to detect genotoxic chemicals which may have different mechanisms of action.

The Ames mutagenicity assay measures the ability of chemical agents to induce mutations in certain strains of bacteria. The suspect chemicals are tested with five specially constructed mutants of *Salmonella typhimurium*, selected for sensitivity and specificity in being reverted from a histidine requirement back to prototrophy by chemical mutagens. By adding homogenate of rat liver (S9) to the plates, thus incorporating an aspect of mammalian metabolism *in vitro*, the assay may also detect potential mutagens which require metabolic activation.

The CHO/HGPRT assay measures the ability of a test compound to induce forward mutations at the enzyme hypoxanthine-guanine phosphoribosyl-transferase (HGPRT) locus of Chinese hamster ovary cells on the basis that presumptive mutants which are defective in the enzyme HGPRT are unable to convert purine analogues such as 6-thioguanine to toxic metabolites. Hence, in a selection medium containing 6-thioguanine, the mutant cells will be able to grow, while the wild type cells are killed.

The assay for sister chromatid exchange (SCE) in Chinese hamster ovary (CHO) cells measures the ability of an agent to increase SCEs above an established baseline. SCEs are detected from the differential staining of chromatids in cytological preparations of metaphase chromosomes. These changes presumably involve DNA breakage and reunion and are therefore thought to be indications of DNA damage.

Detection of chromosome aberrations (CA) is a classical method of assessing the effect of physical and chemical agents on the genetic apparatus of cells. Alterations in chromosomes of CHO cells as a result of exposure to a test agent are visualized in stained cytological preparations of metaphase chromosomes. The genetic consequences of SCE and CA are not known.

The BALB/c-3T3 neoplastic transformation assay is designed to measure the ability of chemical agents to induce alterations in a population of cells (derived from mouse embryo fibroblasts) from a pattern of controlled monolayer growth to one exhibiting foci of disoriented, piled up growth against the background monolayer cells. In vitro transformation is based on morphological events that mimic oncogenesis in vivo. Transformed cell populations in general assume other properties of tumor cells such as growth in semi-solid medium, and often, tumorigenicity in syngeneic, immunosuppressed host animals.

MATERIALS AND METHODS

TEST COMPOUNDS

The compound dimethyl methylphosphonate (DMMP) (lot number 061547), was purchased from Aldrich Chemical Company. Data provided by the supplier showed that the purity of the chemical is better than 99% by gas chromatography. The other test compound exo-tetrahydrodi(cyclopentadiene) (JP-10) was provided by the U.S. Air Force. Some of the physiochemical properties of these two compounds are listed in Table 1. The stability of the test compounds during the treatment periods of the various assays is not known. The positive control compounds ethylmethanesulfonate (EMS) and 3-methylcholanthrene (MCA) were provided by the National Cancer Institute through IITRI, 2-aminoanthracene, 2-nitrofluorene were obtained from Aldrich Chemical Company and 9-aminoacridine and sodium azide were from Sigma Chemical Company. These control compounds are stored in a -20°C freezer or a 4°C refrigerator designated for hazardous substances. The test compounds DMMP and JP-10 are stored at room temperature in a safety cabinet. All compounds were used as received without further chemical analysis. They were weighed and diluted immediately prior to use on the day of the experiment.

SALMONELLA/MAMMALIAN-MICROSOMAL MUTAGENICITY ASSAY (AMES)

Salmonella Tester Strains

The Salmonella typhimurium strains used in this study were obtained from Dr. Bruce Ames, University of California, Berkeley, California, and are identified as TA-98, TA-1538, TA-100, TA-1535, and TA-1537. Their properties and specific details of the assay have been described by Ames and co-workers (Ames et al., 1973). Master cultures from which working cultures

TABLE 1

PHYSICOCHEMICAL PROPERTIES OF THE TEST COMPOUNDS

Chemical Name	Dimethyl methyl-phosphonate DMMP	Exo-tetrahydrodi (cyclopentadiene) JP-10
Chemical State	Liquid	Liquid
Molecular Formula	$C_3H_9O_3P$	Hydrocarbon
Molecular Weight	124.09	
Liquid Density	1.145 g/cc @ 20°C	0.94 g/cc @ 16°C
Boiling Point	181°C	182°C
Vapor Pressure	0.87 mm Hg @ 25°C	< 5 mm Hg @ 25°C
Volatility	4100 mg/m^3	
Flash Point	43°C	54°C

are prepared are maintained frozen in liquid nitrogen. Working cultures are maintained at -80°C. Confirmation of strain performance is conducted every six months.

Ames Assay Test Procedure

The Ames assay was conducted according to our standard operating procedure #CB/M-812a. A liquid pre-incubation modification (standard operating procedure #CB/M-818) was used to test JP-10 which was not compatible with the standard plate-incorporation assay since it formed oil-like droplets on the surface of the top agar layer.

DMMP was assayed in the standard plate incorporation assay with and without metabolic activation (Aroclor 1254 induced rat liver microsomal fraction, S-9) in two separate experiments at doses of 200 to 0.16 ul/plate. The test organism (0.1 ml), the appropriate sample dilution (0.2 ml) and the S-9 mixture (0.5 ml, if required) were added to 2.0 ml of 0.6% molten top agar containing the histidine/biotin supplement. The molten top agar mixture (in duplicate) was mixed by vortexing and poured on minimal glucose agar plates. The solvent control was the highest volume of DMSO used in each experiment. Positive controls are listed below and included compounds which do and do not require metabolic activation.

Tester Strain	Positive Control Chemicals
All Strains	2-Aminoanthracene, 10 µg/plate
TA-98, TA-1538	2-Nitrofluorene, 10 µg/plate
TA-100, TA-1535	Sodium azide, 10 µg/plate
TA-1537	9-Aminoacridine, 50 µg/plate

After incubation for 48 hours at 37°C, mutant colonies were counted and results reported as (average) total number of revertants per plate ± the standard error of the mean.

JP-10 which was immiscible with the top agar overlay was assayed using a preincubation modification of the Ames assay which alters the conditions of exposure of tester strains to test chemicals to enhance exposure. As for the standard assay, bacteria (0.05 ml), the metabolic activation mixture (0.5 ml) or saline (for assays in the absence of S-9) and the test substances (0.05 ml or less) were combined in a test tube. Before the addition of 2.0 ml of top agar, however, the tubes containing bacteria, enzymes and test material were incubated at 37°C for 30 minutes with end-over-end rotation. At that time, the 2.0 ml of top agar was added and the top agar layer was plated in the usual way. The doses of positive control chemicals per plate were as follows: 2-aminoanthracene, 25 ug; 2-nitrofluorene, 5 µg; sodium azide, 1 ug; and 9-aminoacridine, 50 µg.

Quantitation of Data

The mean number of revertants and the standard error of the mean were calculated for each condition and the results expressed as mean number of revertants per plate.

Acceptability of Assays and Criteria for Response

The criteria used to determine the validity and results of an assay include the following:

- Solvent control values must be within the normal range.
- Number of revertants induced by positive control chemicals must be within the historical normal range.
- There must be a healthy (background) "lawn" of cells indicating that the test chemicals have not been assayed at concentrations that are so cytotoxic that all induced mutants have been killed.
- Concentrations of materials tested should include toxic doses since mutagenicity and toxicity are related, but non-toxic doses must also be tested.
- A twofold increase in numbers of revertants over the spontaneous number of revertants with a positive dose-response relationship is considered a positive test.
- Dose-response curves should be reproducible.
- A positive dose-response relationship which does not double the number of spontaneous revertants is an indication of an inconclusive (±) test.

CELL CULTURES

The cells used in the mutagenesis and cytogenetic studies were subclone BH_4 of strain K_1 of the Chinese hamster ovary (CHO) cell line. The stock cultures were originally obtained from Dr. Abraham Hsie's Laboratory (Oak Ridge National Laboratory, Oak Ridge, Tennessee) in April, 1982, and stored in liquid nitrogen. A quality control monitoring of CHO cells prior to the CHO/HGPRT mutagenesis assay indicated that our stock cells had an acceptable spontaneous mutation frequency of $<10^{-6}$ per clonable cell.

The CHO cells were maintained in Ham's F12 medium supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin and 100 µg/ml streptomycin. They were grown in monolayer culture at 37°C in an atmosphere of 5% CO_2 and 95% humidity. For subculture, the cells were detached with a 0.05% trypsin/-0.02% EDTA solution. Under these culture conditions, the cells have a doubling time of approximately 12 hours and maintain a stable karyotype of 19-20 chromosomes.

The cells used in the *in vitro* transformation assay were BALB/c-3T3 cells from a mouse fibroblast cell line. The original cell stock of BALB/c-3T3 clone 1-13 cells were obtained from Dr. T. Kakunaga, National Cancer Institute, in January, 1977. These cells were expanded in culture and stored in liquid nitrogen in sealed ampules at 10^6 cells/ampule. Each working stock of target cells, consisting of 50-60 ampules of cells (k series), was expanded from a frozen ampule of the original stock cells from

Dr. T. Kakunaga. The medium used in the assay was Eagle's minimal essential medium supplemented with 10% fetal calf serum; penicillin and streptomycin were employed at 50 units/ml and 50 μg/ml, respectively.

TREATMENT CONDITIONS

In some of the early experiments, DMMP and JP-10 were assumed to be volatile. Thus, for the cytotoxicity and the cytogenetic assays on DMMP, as well as two cytotoxicity assays on JP-10 (Method 1), the target cells (CHO) were exposed to the test chemicals in sealed glass chambers at 37°C in medium supplemented with 0.02M HEPES buffer, pH 7.4. The brief 24-hour incubation period in glass chamber had no apparent adverse effect on the CHO cells. In the course of the study, it was found that DMMP and JP-10 would have minimum volatility under the incubation conditions of the various assays. All subsequent assays were conducted in the standard CO_2 incubator conditions.

CHO/HGPRT MUTAGENESIS ASSAY

Cytotoxicity Determination

For the CHO/HGPRT mutagenesis assay, the cytotoxic effect of DMMP and JP-10 to CHO cells was determined by a direct cloning assay which measured a reduction in colony-forming ability of the cells following a 24-hour treatment with the test compound. Cells were plated at 300 cells per 100 mm tissue culture dish 24 hours prior to treatment with 0.1 - 1000 μg/ml of DMMP or JP-10.

With compound JP-10, sample delivery was conducted by two methods. In the standard method, appropriate stock concentrations of the test sample were added in 0.2 ml aliquots to culture dishes containing 10 ml of medium (method 1). Using this method, the higher concentrations of JP-10, at 100 and 1000 μg/ml, were found not to be miscible with the culture medium, but appeared as oil-like droplets floating on top of the medium. A second method of sample delivery was therefore used. In this method the final concentration of sample was directly prepared in the culture medium which was then added to the cells (method 2).

After 24 hours of treatment, the medium was removed, the cells were rinsed two times with PBS, and fresh medium was added. The cells were incubated for 7 days to allow colonies to develop, then rinsed with PBS, fixed in 100% methanol and stained with 5% Giemsa. Colony counts were made and cloning efficiencies and surviving fractions determined. The selection of concentrations for the CHO/HGPRT mutagenesis assay was based on the results of this direct cloning assay.

CHO/HGPRT Test Procedure

Mutation induction at the HGPRT locus was measured using our standard operating procedure #CB/M-806 for this assay. Briefly, cells were plated at 5×10^5 cells per 100 mm dish in Ham's F12 medium containing 5% dialyzed fetal calf serum (FCM). After 24 hours, duplicate cultures were treated with DMMP or JP-10, the primary stocks of which were prepared in DMSO. The solvent control was the highest concentration of DMSO used in the experiment

and the positive control ethylmethanesulfonate (EMS) was used at 248 μg/ml. The cells were treated for 16 hours after which the medium was removed, the cells were rinsed twice with PBS, and fresh Ham's F12 medium containing 5% FCM was added. After 24 hours of incubation, the cells from each dish were trypsinized, counted, and plated in duplicate at 1×10^6 cells per 100 mm dish in 10 ml of Ham's F12 medium containing 5% FCM. To allow for phenotypic expression, 1×10^6 cells were subcultured from each set of dishes every 2-3 days for 10 days. The cells were then plated for selection of 6-thioguanine resistant mutants. Cells from duplicate plates were trypsinized, pooled, and plated at a concentration of 2×10^5 cells per 100 mm dish (5 dishes per set) in 10 ml of Ham's F12 medium without hypoxanthine and with 10 μM 6-thioguanine. From the same stock cells, duplicate 60 mm dishes were plated with 200 cells in 5 ml of Ham's F12 medium with 5% FCM for determination of cloning efficiency.

After 7 days of incubation, the 60 mm dishes for cloning efficiency determinations were rinsed with PBS, fixed in methanol, and stained with 5% Giemsa. The same was done with the 100 mm dishes for mutant colonies, after 9 days of incubation. Colony counts were made and cloning efficiencies and surviving fractions determined by pooling the results of duplicate sets.

Quantitation and Statistical Analysis of Data

The mutation frequency was calculated by dividing the total number of mutant colonies by the total number of cells plated (2×10^6) corrected for the cloning efficiency. Mutation frequency was then expressed as the number of mutants per 10^6 clonable cells. To determine the statistical significance of the mean number of mutant colonies per plate of the treated condition versus the control, the following two-tailed t-test was carried out.

$$s = \sqrt{\frac{(n_c-1)s_c^2 + (n_t-1)s_t^2}{n_c + n_t - 1}}$$

$$t = \frac{|\bar{x}_c - \bar{x}_t|}{s\sqrt{\frac{1}{n_c} + \frac{1}{n_t}}}$$

n_c = sample size of control population;

n_t = sample size of test population;

s_c = standard deviation of control population;

s_t = standard deviation of test population;

x_c = mean of control population;

x_t = mean of test population.

A value of $p<0.05$ was considered statistically significant.

Acceptability of Assay and Criteria for Response

The following criteria were used to evaluate the assay results:

- The cloning efficiency of CHO cells is 60% or better.
- The spontaneous frequency is between 0-20 mutants per 10^6 clonable cells.
- The positive control, EMS at 248 μg/ml induced at least 300 mutants per 10^6 clonable cells.
- An agent is considered positive in the assay if it induces a statistically significant ($p<0.05$) mutation frequency above the control, (or solvent control, whichever has a higher frequency) and responds in a dose-dependent manner.

CYTOGENETIC ASSAYS

Cytotoxicity Determination

The cytotoxicity of DMMP and JP-10 to CHO cells was determined by a reduction in cell number following a 24-hour treatment of cells in mass culture. Cells were plated at 3×10^5/100 mm dish 24 hours prior to treatment and exposed to test chemical the following day at concentrations varying from 0.1 - 1000 μg/ml. As in the cytotoxicity determination for the HGPRT assay, the sample delivery of JP-10 was conducted by two different methods. After a 24 hour exposure period, the medium containing test chemical was removed, the cells were rinsed three times with phosphate buffered saline (PBS), trypsinized and counted. The ratio of the cell counts of the treated cultures to that of the untreated control was calculated for each concentration of chemical tested. The selection of doses for the cytogenetic assays (SCE and CA) was based on these results. An untreated control, a solvent control and a positive control of ethylmethanesulfonate (EMS) at 124 μg/ml for the SCE assay or 248 μg/ml for the CA assay were included.

Sister Chromatid Exchanges (SCE) Test Procedure

Sister chromatid exchanges were examined in CHO cells using our standard operating procedure #CB/M-804. Cells were plated at 3×10^5/100 mm dish containing 10 ml of Ham's F-12 medium with 10% FCS. The following day, cells were treated with the test chemical. At the time of treatment, bromodeoxyuridine (BrdU) at a final concentration of 2.5×10^{-5}M was also added to the cultures. After the addition of BrdU, the cells were protected from light and incubated at 37°C.

Twenty-two hours after the initiation of treatment with the test compound and BrdU, Colcemid, at a final concentration of 0.45 μg/ml was added for the last two hours of culture. The cells were then harvested by trypsinization and combined with the culture medium which could contain dividing cells. The cell suspensions were centrifuged and the cell pellets were resuspended in 0.075 M KCl for 7-10 minutes, centrifuged, and fixed in

3:1 methanol and acetic acid. The fixative was changed twice before slides were made by dropping the suspension on clean slides and air-drying. The slides were stained by the fluorescence-plus-Giemsa technique (standard operating procedure #CB/M-515).

A total of 50 metaphases were scored microscopically for each experimental condition (25 from each of the duplicate cultures), and chromosome counts were made on each metaphase scored. Mitotic index was determined by counting 2000 randomly selected cells from each experimental condition and the number of cells which were undergoing mitosis was expressed as percentage of the total cells counted.

Quantitation and Statistical Analysis of Data

The mean number of SCE, standard deviation, and standard error of the mean were calculated for each condition and the results were expressed as the number of SCE per cell. To determine statistical significance of the treated condition above the control a two-tailed t-test like that used in the HGPRT assay was carried out.

Acceptability of Assay and Criteria for Response

The following criteria were used to evaluate the assay results:

- A minimum of 30 metaphases must be scored for each test condition in at least two of the four doses tested.

- The controls (negative and positive) must have the acceptable number (30 or more) of metaphases scored.

- The positive control induced a statistically significant ($p<0.01$) increase in SCE above the untreated or solvent control, whichever has the higher frequency.

- An agent is considered positive in the assay if it induces a statistically significant ($p<0.05$) SCE frequency above the control (or solvent control) and responds in a dose-dependent manner.

Chromosome Aberrations Test Procedure

The experimental design for the chromosome aberrations assay (standard operating procedure #CB/M-805) was similar to that of the SCE assay, and the two were performed simultaneously in parallel cultures. At the time of chemical treatment no BrdU was added to the plates for chromosome aberrations. The methods for harvesting the cells and making slides are identical to those described previously for the SCE assay, however, the staining procedure was different. For the chromosome aberrations assay, air-dried slides were simply stained with 4% Giemsa for 6 minutes, rinsed in distilled water and air-dried.

One hundred metaphases from each experimental condition (50 from each of the duplicate cultures) were scored microscopically for chromosome aberrations. The following classifications of aberrations were scored:

1. Chromosome aberrations - changes involving whole chromosomes and observed at homologous sites of both chromatids of a chromosome. These include breaks, fragments, markers (i.e., dicentrics, rings), and gaps.

2. Chromatid aberrations - changes involving individual chromatids of a chromosome. These include breaks, fragments, interchanges (i.e., triradials, quadraradials), and gaps.

3. Numerous aberrations - changes involving many or all chromosomes within a cell. Pulverized chromosomes and cells with greater than ten aberrations are classified in this group.

In all three classifications gaps were scored but not included in the final calculations of the results.

Quantitation and Statistical Analysis of Data

Chromosome counts were made on each metaphase scored, and the mitotic index was also determined from the slides. The data were tabulated and expressed as the mean number of chromosome aberrations per cell and the percentage of cells with chromosome aberrations. Statistical analyses were performed as described in the HGPRT assay.

Acceptability of Assay and Criteria for Response

The following criteria were used to evaluate the assay results:

- A minimum of 60 metaphases must be scored for each test condition to be included in the calculation. At least two of the four doses tested must have the acceptable number of metaphases scored.

- The controls (negative and positive) must have the acceptable number (60 or more) of metaphases scored.

- An agent is considered positive if it induces a statistically significant ($p<0.05$) increase in aberration frequency above the control in at least two of the four concentrations tested. A dose-dependent effect is corroborative evidence of a positive response. The following guideline may also be used in the judgment of a positive response.

Response	% Cells with Aberrations
-	< 4.9%
±	5.0 - 9.9%
+	10.0 - 19.9%
++	20.0 - 49.9%
+++	> 50.0%

BALB/c-3T3 CELL TRANSFORMATION ASSAY

Cytotoxicity Determination

A prescreening of the cytotoxicity of the test chemical was conducted to determine the highest concentration of the test chemical to be used in the transformation assay. The target cells which were plated at 5×10^3/well in cluster dishes were treated with test chemical the following day. Five concentrations of the chemical varying from 0.01-100 µg/ml were tested. The surviving fraction of cells treated with chemical as compared to untreated cells was determined after a 3-day exposure (the exposure time for the assay). The highest concentration of chemical used in the transformation assay was based on the results of this cytotoxicity test. For each assay 4 concentrations of the chemical in serial dilution factor of 0.2 were used. An untreated control and a positive control of 3-methylcholanthrene (MCA) at 2 µg/ml were included in the assay.

In Vitro Transformation Test Procedure

The transformation assay was performed as described in our standard operating procedure #CB/M-816. Dishes for transformation assay were plated with cells expanded from frozen stock at 10^4 cells/plate. Twenty-four hours later, the test chemicals were added to the appropriate plates. After a three day treatment, the medium was removed, the plates washed and replenished with fresh medium (without chemicals) and incubated for approximately four weeks. During this incubation period, the medium was replaced every 4-7 days. At the end of the incubation period, the plates were fixed with methanol and stained with 2-3% Giemsa stain. Type III foci (Resnikoff et al., 1973) were counted in each plate using a dissecting microscope. Type III foci are aggregations of fibroblastic cells that are highly polar, multilayered and randomly oriented exhibiting a criss-crossed, densely stained basophilic array.

The cytotoxicity of the test chemical on BALB/c-3T3 cells was determined in parallel with the transformation assay. Cells of two randomly chosen plates from each experimental condition were trypsinized and counted at the end of the 3-day treatment period. The cloning efficiency of these cells was determined by replating the counted cells at 100 cells/plate. The plates were incubated for 17 days, fixed with methanol, stained with 4-5% Giemsa and the colonies were counted.

Quantitation and Statistical Analysis of Data

The mean number of Type III foci/plate, the fraction of plates with Type III foci, and the standard error of the mean for each experimental set were calculated. To test if the number of foci/plate of the treated sets is significant ($p<0.05$) above that of the control, an approximate t-test was carried out.

$$t \cong \frac{\hat{\lambda}_T - \hat{\lambda}_C}{\sqrt{\frac{S_T^2}{n'_T} + \frac{S_C^2}{n'_C}}}$$

$$df = \frac{\left[\frac{S_T^2}{n'_T} + \frac{S_C^2}{n'_C}\right]^2}{\frac{1}{n'_T}\left[\frac{S_T^2}{n'_T}\right]^2 , \left[\frac{S_C^2}{n'_C}\right]^2 \frac{1}{n'_C}}$$

$\hat{\lambda}$ is the mean foci/plate

n' is the number of plates

The approximate t-test uses a table of critical t-values for a none tailed $p<0.05$.

Acceptability of Assay and Criteria for Response

The following criteria were used to evaluate the assay results:

- A minimum of 10 plates must be scored for each test condition to be included in the calculation.

- Three of the four doses of test compound must have the minimum acceptable number of plates scored.

- The controls (negative and positive) must have the acceptable number of plates scored.

- The upper limit of the Type III foci/plate for the untreated control is 0.75. The lower limit of Type III foci/plate for the positive control (MCA, 2 μg/ml) is 1.20.

- An agent is considered positive in the assay if it induces a statistically significant ($p<0.05$) increase in Type III foci/plate above the untreated control in at least two of the four concentrations tested. The fraction of plates with foci and a positive dose-response are corroborative data used in the final judgment of a positive response.

RESULTS

DIMETHYL METHYLPHOSPHONATE (DMMP)

Ames Assay

DMMP was assayed by the standard plate incorporation Ames Assay in two experiments. In both experiments all positive control chemicals elicited a positive response and DMSO control backgrounds were acceptable. In the first experiment (Table 2), the doses were 200 to 5 μl per plate. At the higher doses, DMMP was toxic as evidenced by the sparseness of the background bacterial lawn. Even at the lowest dose tested, DMMP toxicity was evident by the fact that the number of revertants obtained per plate were smaller than those seen in the negative control plates. Although no evidence of mutagenicity was seen with or without metabolic activation, an assay on DMMP was repeated at lower concentrations (Table 3). Toxicity was evident at the highest concentration tested (100 μl/plate) and no mutagenic activity was detected at the lower concentrations.

CHO/HGPRT Assay

Data on the cytotoxicity of DMMP to CHO cells are presented in Table 4. Over a five-log range of concentrations tested, no cytotoxic effect of DMMP was evident. The highest concentration used in the mammalian cell gene mutation assay was 1000 μg/ml.

Results of the HGPRT mutagenesis assay (Table 5) show that a similar number of total mutant colonies were scored in the control and DMMP-treated conditions. This clearly demonstrates that DMMP induced no mutagenic response at the HGPRT locus above that of the spontaneous events. A technical error made in assessing the cloning efficiencies of the CHO cells at the mutant selection time precluded calculation of the exact mutation frequency. An estimation was made based on previous data that DMMP was not cytotoxic.

Cytogenetic Assays

For the cytogenetic analysis of SCE and CA, the cytotoxic effect of DMMP was determined by a reduction in relative number of CHO cells in mass culture. Under these conditions, DMMP was also not cytotoxic (Table 6) as was also observed in the more stringent viability criteria of clonal growth determined in the HGPRT assay. Therefore, the same concentrations tested in the gene mutation assay were also used for the SCE and CA assays.

The effects of DMMP on SCE and CA of the CHO cells are shown in Tables 7 and 8 respectively. The mean SCE/cell found in the DMMP-treated CHO cells at the concentrations tested was not significantly higher than the solvent control of 1% DMSO. Only the positive control (EMS) induced a statistically significant increase in SCE and a reduction in mitotic index of the CHO cells. In contrast, DMMP induced a statistically significant increase in chromosome aberrations at 250 ($p<0.05$) and 1000 ($p<0.01$) μg/ml but not at the intermediate 500 μg/ml or the low concentration of 125 μg/ml. Thus, DMMP appeared to have a low clastogenic activity.

TABLE 2

AMES ASSAY

DIMETHYL METHYLPHOSPHONATE (Experiment 1)

	Revertants Per Plate[a]									
	TA-98		TA-1537		TA-1538		TA-100		TA-1535	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Negative Control[b]	16±2	24±2	11±1	8±1	12±2	22±5	114±2	103±7	16±4	9±1
2-Aminoanthracene[b] (10 µg/plate)	32±7	TNTC	12±2	TNTC	14±0	TNTC	171±5	TNTC	25±6	189±41
2-Nitrofluorene[b] (10 µg/plate)	TNTC	TNTC			TNTC	TNTC				
9-Aminoacridine[b] (50 µg/plate)			TNTC	213±37						
Sodium Azide[c] (10 µg/plate)							TNTC	TNTC	TNTC	TNTC
Dimethylmethyl phosphonate										
(200 µl/plate)	97±0[d]	16±1	10±1	3±1[d]	0[d]	5±1	41±0[d]	176±8	134±50[d]	18±9[d]
(100 µl/plate)	12±1	20±2	2±2	3±1[d]	d	7±4[d]	98±8	156±5	11±2	9±1[d]
(50 µl/plate)	14±2	28±1	3±1	7±1	9±3	14±2	125±3	148±8	27±4	19±1
(10 µl/plate)	14±1	37±1	8±1	9±1	11±1	24±1	110±1	110±6	25±7	10±1
(5 µl/plate)	15±1	23±2	8±0	10±2	8±3	15±1	95±15	102±6	19±1	13±2

[a]Mean of two replicate plates ± standard error of the mean

[b]Dissolved/diluted in dimethylsulfoxide (DMSO)

[c]Dissolved/diluted in sterile distilled water

[d]Toxic effect seen

TABLE 3

AMES ASSAY

DIMETHYL METHYLPHOSPHONATE (Experiment 2)

	Revertants Per Plate[a]									
	TA-98		TA-1537		TA-1538		TA-100		TA-1535	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Negative Control[b]	23±2	28±7	15±1	9±1	14±7	26±3	148±5	140±6	48±8	11±1
2-Aminoanthracene[b] (10 µg/plate)	33 9	TNTC	18 2	118 18	16 1	141 10	159 5	TNTC	49 2	101 3
2-Nitrofluorene[b] (10 µg/plate)	TNTC	TNTC			TNTC	TNTC				
9-Aminoacridine[b] (50 µg/plate)			TNTC	161±41						
Sodium Azide[c] (10 µg/plate)							TNTC	TNTC	TNTC	TNTC
Dimethylmethyl phosphonate										
(100 µl/plate)	8±1[d]	24±3	2±1	5±2	d	13±1	149±12	154±1	23±4	13±1
(20 µl/plate)	20±3	28±3	9±2	16±4	8±1	28±3	167±2	165±2	38±2	13±0
(4 µl/plate)	17±2	27±3	8±2	9±2	10±1	21±3	143±9	125±3	36±2	17±3
(0.8 µl/plate)	14±4	34±7	8±3	9±1	13±2	19±4	137±8	129±11	43±8	14±1
(0.16 µl/plate)	17±2	31±6	8±2	13±1	15±7	27±5	148±2	121±13	35±2	11±2

[a]Mean of two replicate plates ± standard error of the mean

[b]Dissolved/diluted in dimethylsulfoxide (DMSO)

[c]Dissolved/diluted in sterile distilled water

[d]Toxic effect seen

TABLE 4

CYTOTOXICITY OF DIMETHYL METHYLPHOSPHONATE ON CHO CELLS
CLONAL DETERMINATION

Concentration (µg/ml	Colonies/Plate ± S.E.M.[a]	Treated/Control
0	250 ± 36.3	-
0.1	313 ± 10.3	1.25
1.0	320 ± 11.0	1.28
10.0	299 ± 8.6	1.20
100.0	298 ± 42.3	1.19
1000.0	288 ± 7.0	1.15
Solvent Control (2% DMSO)	275 ± 18.8	1.10

[a]Plating density: 300 cells/100mm plate; mean of 4 plates ± standard error of the mean.

TABLE 5

THE EFFECT OF DIMETHYL METHYLPHOSPHONATE ON HGPRT MUTATIONS IN CHO CELLS

Chemical	µg/ml	Total Colonies[a]	% Cloning Efficiency	Mutation Frequency[b]
Control	0	10	-	5.00[c]
DMMP	125	12	217	2.76
	250	6	113	2.65
	500	8	133	3.01
	1000	7	135	2.59
EMS	248	1309	-	654.50[c]
Solvent Control (1% DMSO)	0	5	-	2.50[c]

[a] Plating Density: 2×10^5 cells/plate, a total of 10 plates used.

[b] Mutation frequency is expressed as number of mutants/10^6 clonable cells.

[c] Due to technical error cloning efficiency could not be determined. Mutation frequency calculated by assuming a cloning efficiency of 100% based on results of previous experiments.

TABLE 6

CYTOTOXICITY OF DIMETHYL METHYLPHOSPHONATE ON CHO CELLS
MASS CULTURE DETERMINATION

Concentration (μg/ml)	Cells/plate[a] x 10^6	Treated/Control
0	1.54	-
0.1	1.73	1.12
1.0	1.92	1.27
10.0	2.00	1.30
100.0	2.11	1.37
1000.0	1.77	1.15
Solvent Control (2% DMSO)	1.23	0.79

[a]Plating density: 3 x 10^5 cells/100mm plate; mean of 2 plates.

TABLE 7

THE EFFECT OF DIMETHYL METHYLPHOSPHONATE ON SISTER-CHROMATID EXCHANGE IN CHO CELLS

Chemical	µg/ml	Total SCE[a]	Mean SCE/Cell ±S.D.[b]	Total Chromosomes[a]	Mean Chromosome/ Cell ± S.D.[b]	Mitotic Index[c]
Control	0	740	14.80±4.42	988	19.76±0.80	6.8
Solvent Control (1% DMSO)	0	889	17.78±4.22	982	19.64±0.92	5.9
DMMP	125	719	14.38±3.88	974	19.48±0.93	8.4
	250	854	17.08±4.45	974	19.48±0.86	8.6
	500	825	16.50±3.60	978	19.56±0.88	6.1
	1000	922	18.44±5.02	987	19.74±1.08	7.5
EMS	124	2089	41.78±9.08	993	19.86±1.09	1.9

[a] A total of 50 cells were scored for each test condition (25 cells from each duplicate cultures).

[b] S.D. = Standard deviation.

[c] The percentage of cells undergoing mitosis determined by counting 2000 cells randomly selected.

TABLE 8

THE EFFECT OF DIMETHYL METHYLPHOSPHONATE ON CHROMOSOME ABERRATIONS IN CHO CELLS

	Solvent Control (0.2% DMSO)	Control	DMMP Concentration (µg/ml) 125	250	500	1000	EMS (µg/ml) 248
Chromosome Aberrations							
Markers	0	0	2	3	0	1	3
Breaks	0	0	1	0	3	0	10
Fragments	0	0	0	1	0	3	4
Gaps[a]	0	0	0	1	3	1	1
Chromatid Aberrations							
Interchanges	0	0	0	0	0	0	5
Breaks	1	0	0	2	0	6	11
Fragments	1	0	1	0	0	3	1
Gaps[a]	4	3	1	4	9	0	8
Numerous Aberrations							
Pulverized	0	0	0	0	0	0	0
>10 Aberrations	0	0	0	0	0	0	0
Total Aberrations	2	0	4	6	3	13	34
Aberrations/Cell±S.E.M.[b]	0.02±0.014	0.00±0.000	0.04±0.024	0.06±0.028	0.03±0.017	0.10±0.030	0.34±0.064
% Cells with Aberrations	2	0	3	5	3	10	26
Mitotic Index[c]	6.3	6.3	6.1	6.6	5.6	5.8	4.4

[a] Gaps were scored but not included as aberrations in the final calculations.

[b] A total of 100 cells were scored for each test condition (50 cells from each of the duplicate cultures). S.E.M. = Standard error of the mean.

[c] The percentage of cells undergoing mitosis determined by counting 2000 cells randomly selected.

BALB/c-3T3 Transformation Assay

Although non-toxic on CHO cells, DMMP induced some cytotoxicity on the mouse BALB/c-3T3 cells. In the mass culture screening assay used for dosage selection, the relative surviving fraction of BALB/c cells was 0.30 when treated with 100 μg/ml of the chemical for 3 days (Table 9). This was the highest concentration used in the transformation assay. The results in Table 10 show that DMMP did not induce transformation under conditions of the assay.

EXO-TETRAHYDRODI-(CYCLOPENTADIENE) (JP-10)

Ames Assay

JP-10 was assayed by a preincubation modification of the Ames assay because it was immiscible with the agar medium used in this assay. Varying volumes of the undiluted test material were added directly to the bacteria and S-9 (or saline) mixes for preincubation because JP-10 was not sufficiently soluble in DMSO to obtain the necessary concentrates for the desired doses. Even at the lowest dose tested (1 μl/plate) toxicity was severe enough to result in sparse background lawns. Many plates had no revertants rather than the number normally obtained as a result of spontaneous reversion (Table 11).

The results of this assay are inconclusive due to severe toxicity of JP-10 on the tester strains. Although a repeat assay with shorter exposure times is recommended, we consider that further modifications of the standard protocol for this particularly toxic test substance are outside the scope of this present work assignment.

CHO/HGPRT Assay

In the initial cytotoxicity assays conducted with JP-10, stock concentrations of the test compound were delivered in small volumes (0.2 ml) to CHO cells in 10 ml of culture medium. With this sample delivery method, it was noted that the higher concentrations of JP-10 (100 and 1000 μg/ml) appeared not to be miscible with the culture medium. Results of the cytotoxicity determination with JP-10 by the clonal growth method showed that JP-10 was not cytotoxic up to 1,000 μg/ml, the highest concentration tested (Table 12). However, when the CHO cells were exposed to the test sample by replacing medium with medium containing the final concentrations of the test compound, JP-10 was very cytotoxic to the cells (Table 13). No clonal growth was found in cells treated with 1 μg/ml of JP-10.

Four concentrations of JP-10, starting from 1.0 μg/ml, in two-fold dilution, were tested in the mammalian gene mutation assay. The results in Table 14 show that JP-10 did not induce HGPRT mutations at the concentrations tested. In contrast to the earlier cytotoxicity assay, 1.0 μg/ml of JP-10 was not toxic in the mutation assay. This variability in cytotoxic effect of JP-10 reflects a solubility problem in aqueous medium with this compound. In another experiment, JP-10 also did not induce any mutagenic response even at 125 μg/ml, when cytotoxicity was evident (data not shown).

TABLE 9

CYTOTOXICITY OF DIMETHYL METHYLPHOSPHONATE ON BALB/C-3T3 CELLS
MASS CULTURE DETERMINATION

Concentration (μg/ml)	Cells/Plate[a] x 10^5	Treated/Control
0	7.0	-
0.01	5.4	0.77
0.1	4.2	0.59
1.0	5.6	0.80
10.0	4.5	0.64
100.0	2.1	0.30

[a]Plating density: 5 x 10^3 cells/35mm well; mean of 2 wells.

TABLE 10

BALB/c-3T3 TRANSFORMATION ASSAY ON DIMETHYL METHYLPHOSPHONATE

	Control	Concentration (μg/ml) 0.8	4.0	20.0	100.0	MCA (2 μg/ml)
Total Foci/ Total Plates	0/19	2/20	2/20	1/15	1/20	26/19
Foci per Plate ± Std. Error of Mean	0	0.10 ± 0.07	0.10 ± 0.07	0.07 ± 0.06	0.05 ± 0.05	1.37 ± 0.26
Total Plates with Foci/Total Plates	0/19	2/20	2/20	1/15	1/20	14/19
Ratio	0	0.10	0.10	0.07	0.05	0.74
T-Statistic		1.41	1.41	1.02	1.00	5.10
Degrees of Freedom		20	20	15	20	19
Significant Transformation Activity ($\alpha = 0.05$)		No	No	No	No	Yes
Cells/Plate x 10^5	7.6	3.4	4.0	3.2	4.8	2.1
Treated/Control		0.45	0.53	0.42	0.63	0.28
Clones/100 Cells	31.0	13.8	10.4	3.6	9.8	18.4
Treated/Control		0.45	0.34	0.12[a]	0.32	0.59

[a]Slightly contaminated

TABLE 11

AMES ASSAY (30 MINUTE PREINCUBATION WITH END OVER END ROTATION)

JP-10

	Revertants Per Plate[a]									
	TA-98		TA-1537		TA-1538		TA-100		TA-1535	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Negative Control[b]	18±3	27±2	10±0	14±1	11±2	24±3	125±11	94±5	32±6	9±4
2-Aminoanthracene[b] (25 µg/plate)	24±2	TNTC	11±2	277±3	11±3	TNTC	136±3	TNTC	33±1	140±8
2-Nitrofluorene[b] (5 µg/plate)	497±17	228±10			TNTC	307±3				
9-Aminoacridine[b] (50 µg/olate)			4±0[d]	TNTC						
Sodium Azide[c] (1 µg/plate)							TNTC	120±10	TNTC	52±13
JP-10										
(50 µl/plate)	e	d	e	d	e	d	e	d	34±2	13±1
(20 µl/plate)	d	d	e	d	d	d	d	d	4±4[d]	5±1[d]
(10 µl/plate)	d	d	e	d	d	d	d	d	d	d
(5 µl/plate)	5±2[d]	d	d	d	d	d	d	d	e	4±4[d]
(1 µl/plate)	8±2	11±2[d]	d	d	d	d	d	94±2	1±0	14±4

[a]Mean of two replicate plates ± standard error of the mean

[b]Dissolved/diluted in dimethylsulfoxide (DMSO)

[c]Dissolved/diluted in sterile distilled water

[d]Toxic effect seen, sparse background

[e]Toxic effect seen, total destruction of lawn

TABLE 12

CYTOTOXICITY OF JP-10 ON CHO CELLS
CLONAL DETERMINATION (METHOD 1[a])

Concentration (μg/ml)	Colonies/plate S.E.M.[b]	Treated/Control
0	245 ± 11.3	-
0.1	267 ± 1.8	1.09
1.0	234 ± 1.2	0.95
10.0	262 ± 4.0	1.07
100.0	258 ± 12.8	1.05
1000.0	253 ± 7.3	1.05
Solvent Control (0.3% DMSO)	251 ± 10.0	1.02

[a]Stock concentrations of JP-10, in volume of 0.2 ml, were added to cells in 10 ml of medium to attain the final concentrations indicated.

[b]Plating density: 300 cells/100mm plate; mean of 2-4 plates ± standard error of the mean.

TABLE 13

CYTOTOXICITY OF JP-10 ON CHO CELLS

CLONAL DETERMINATION (METHOD 2[a])

Concentration (μg/ml)	Colonies/Plate±S.E.M.[b]	Treated/Control[c]
0	68 ± 17.0	-
0.01	165 ± 14.5	0.77
0.1	153 ± 62.5	0.71
1.0	0	0
10.0	0	0
100.0	0	0
Solvent Control (1% DMSO)	215 ± 10.0	1.00

[a]The indicated concentrations of JP-10 were made up in complete medium and added to cells in a final volume of 10 ml.

[b]Plating density: 300 cells/100mm plate; mean of 2 plates.

[c]Treated/control calculated based on solvent control due to unusually low cloning efficiency of the untreated control.

TABLE 14

THE EFFECT OF JP-10 ON HGPRT MUTATIONS IN CHO CELLS

Chemical	µg/ml	Total Colonies[a]	% Cloning Efficiency	Mutation Frequency[b]
Control	0	4	72	2.78
JP-10	0.125	1	54	0.93
	0.250	4	127	1.57
	0.500	7	83	4.22
	1.000	7	82	4.27
EMS	248	585	51	573.53
Solvent Control (1% DMSO)		18	95	9.47

[a]Mutation frequency is expressed as number of mutants/10^6 clonable cells.

[b]S.D. = Standard deviation. Plating density: 2 x 10^5; mean of 10 plates.

Cytogenetic Assay

Tables 15 and 16 show data on the cytotoxicity of JP-10 by mass cell culture determination using the two methods of sample delivery already discussed. Again when the test sample was delivered in a small volume (0.2 ml), JP-10 induced no cytotoxicity (Table 15). A substantial cytotoxic effect was observed when JP-10 was premixed with culture medium prior to exposing to the CHO cells (Table 16).

At the concentrations tested, JP-10 produced no enhancement of SCE (Table 17) and a slight increase in chromosome aberrations (Table 18) in CHO cells at the highest concentration tested (1.0 μg/ml).

BALB/c-3T3 Transformation Assay

Results of the cytotoxicity and the transformation of JP-10 on BALB/c-3T3 cells are presented in Table 19 and 20 respectively. The toxicity profile of JP-10 on BALB/c cells was similar to that on CHO cells, with toxic effect evident between 1 and 10 μg/ml (Table 19). No transformation was induced by JP-10 under the conditions of the assay while the positive control MCA induced an acceptable level of transformation of 2.0 Type III foci/plate (Table 20). The cloning efficiency of BALB/c cells in the serum lot used (GIBCO 24N2117) was low and variable. However, the transformation response of BALB/c cells in this serum lot was judged to be in the acceptable range based on a historical mean of 2.7 Type III foci/plate induced by MCA.

CONCLUSIONS

In vivo toxicological data on DMMP are scant. An LD_{50} of >150 mg/kg in the rat has been reported. This study assessed the *in vitro* genotoxic properties of DMMP in a battery of short-term assay utilizing various endpoints.

In the absence of metabolic activation, DMMP induced no mutagenic response in either the microbial target cells (Ames assay) or the mammalian cells (CHO/HGPRT). It also had no effect in assays which were indicative of DNA damage (SCE assay) or measured neoplastic transformation. DMMP exhibited a low clastogenic activity in inducing chromosomal aberrations at the highest concentration tested (1,000 μg/ml). No dose-dependent response was observed under conditions of the assay. In the presence of a microsomal S-9 metabolic activation system, DMMP again had no mutagenic effect in the Ames Salmonella mutagenicity assay.

The second compound tested in this task, JP-10, in contrast to DMMP, was quite toxic to the various target cells used in the *in vitro* assays, thus limiting the concentrations that could be tested. The highest concentration tested in the various cell culture assays was between 1-5 μg/ml. This test compound also was not very miscible with aqueous medium, a prerequisite condition for all the *in vitro* assays. Under these limiting conditions, JP-10 did not induce a genotoxic response in the CHO/HGPRT, CHO/SCE and BALB/c-3T3 assays. It produced a marginally positive response in the CHO/CA assay at the highest concentration (1 μg/ml) tested. Due to the toxic effect of JP-10, results of the Ames assay on this compound, with and without

TABLE 15

CYTOTOXICITY OF JP-10 ON CHO CELLS
MASS CULTURE DETERMINATION (METHOD 1[a])

Concentration (μg/ml)	Cells/Plate[b] x 10^6	Treated/Control
0	1.01	-
0.1	0.78	0.77
1.0	0.84	0.83
10.0	0.77	0.77
100.0	0.82	0.81
1000.0	0.80	0.80
Solvent Control (0.3% DMSO)	0.79	0.78

[a]Stock concentrations of JP-10, in volume of 0.2 ml, were added to cells in 10 ml of medium to attain the final concentrations indicated.

[b]Plating density: 3 x 10^5 cells/100mm plate; mean of 2 plates.

TABLE 16

CYTOTOXICITY OF JP-10 ON CHO CELLS
MASS CULTURE DETERMINATION (METHOD 2[a])

Concentration (μg/ml)	Cells/plate[b] x 10^6	Treated/Control
0	1.44	-
0.1	1.13	0.78
1.0	1.29	0.90
10.0	0.16	0.11
100.0	0.00	0.00
1000.0	0.00	0.00
Solvent Control (1% DMSO)	1.19	0.83

[a]The indicated concentrations of JP-10 were made up in complete medium and added to cells in a final volume of 10 ml.

[b]Plating density: 3 x 10^5 cells/100mm plate; mean of 2 plates.

TABLE 17

THE EFFECT OF JP-10 ON SISTER CHROMATID EXCHANGE IN CHO CELLS

Chemical	µg/ml	Total SCE[a]	Mean SCE/Cell ± S.D.[b]	Total Chromosomes[a]	Mean Chromosome/ Cell ± S.D.[b]	Mitotic Index[c]
Control	0	828	16.56 ± 4.83	1005	20.10 ± 0.99	7.5
Solvent Control (1% DMSO)	0	808	16.16 ± 4.35	997	19.94 ± 0.79	7.6
JP 10	0.125	783	15.66 ± 4.19	975	19.50 ± 1.04	6.0
	0.25	755	15.10 ± 4.82	965	19.30 ± 1.05	7.5
	0.50	757	15.14 ± 4.40	981	19.62 ± 1.26	5.6
	1.00	723	14.46 ± 3.90	969	19.38 ± 1.05	6.5
EMS	124	2565	51.30 ±10.12	1009	20.18 ± 0.87	3.0

[a] A total of 50 cells were scored for each condition tested (25 cells from each duplicate cultures)

[b] S.D. = Standard Deviation

[c] The percentage of cells undergoing mitosis determined by counting 2000 cells randomly selected.

TABLE 1S

THE EFFECT OF JP-10 ON CHROMOSOME ABERRATIONS IN CHO CELLS

	Solvent Control (1% DMSO)	Control	JP-10 Concentration (μg/ml) 0.125	0.25	0.5	1.0	EMS (μg/ml) 248
Chromosome Aberrations							
Markers	1	1	0	2	2	2	3
Breaks	0	0	0	0	0	0	2
Fragments	0	0	2	5	1	3	10
Gaps	0	0	0	1	3	0	1
Chromatid Aberrations							
Interchanges	0	0	0	0	0	0	9
Breaks	0	0	0	0	0	1	6
Fragments	2	1	0	1	0	1	1
Gaps	3	1	0	0	4	10	11
Numerous Aberrations							
Pulverized	0	0	0	0	1	0	5
>10 Aberrations	0	0	0	0	0	0	0
Total Aberrations[a]	3	2	2	8	4	7	36
Aberrations/Cell ± S.E.M.[b]	0.03±0.017	0.02±0.014	0.02±0.020	0.08±0.044	0.04±0.020	0.07±0.026	0.36±0.080
% Cells with Aberrations	3	2	1	5	4	7	25
Mitotic Index[c]	10.8	13.2	16.9	11.6	6.0	7.0	3.9

[a] Gaps were scored but not included as aberrations in the final calculations.

[b] A total of 100 cells were scored for each test condition (50 cells from each of the duplicate cultures). S.E.M. = Standard error of the mean.

[c] The percentage of cells undergoing mitosis determined by counting 2000 cells randomly selected.

TABLE 19

CYTOTOXICITY OF JP-10 ON BALB/C-3T3 CELLS
MASS CULTURE DETERMINATION (METHOD 2)

Concentration (μg/ml)	Cells/Plate[a] x 10^5	Treated/Control
0	8.2	-
0.01	8.4	1.02
0.1	8.4	1.02
1.0	8.0	0.98
10.0	0.3	0.03
100.0	0	0

[a]Plating density: 5 x 10^3 cells/35mm well; mean of 2 wells.

TABLE 20

BALB/c-3T3 TRANSFORMATION ASSAY ON JP-10

		Concentration (μg/ml)				
	CONTROL	0.04	0.2	1.0	5.0	MCA (2 μg/ml)
Total Foci/ Total Plates	2/19	3/20	1/19	4/20	1/20	40/20
Foci per Plate ± S.E.M.[a]	0.11±0.07	0.15±0.08	0.05±0.05	0.20±0.11	0.05±0.05	2.00±0.27
Total Plates with Foci/Total Plates	2/19	3/20	1/19	3/20	1/20	18/20
Ratio	0.11	0.15	0.05	0.15	0.05	0.90
T-Statistic		0.35	0.65	0.72	0.66	5.81
Degrees of Freedom		39	33	37	33	21
Significant Transformation Activity (α 0.05)		No	No	No	No	Yes
Cells/Plate x 10^5	6.0	3.0	3.4	3.8	5.2	2.4
Treated/Control		0.50	0.57	0.63	0.87	0.40
Clones/100 Cells	14.4	6.6	13.0	11.4	10.4	25.2
Treated/Control		0.46	0.90	0.79	0.72	1.75

[a]S.E.M. = standard error of the mean.

metabolic activation, were inconclusive. Table 21 summarizes the results obtained with the two test compounds.

RECOMMENDATIONS

For this task order, the four mammalian cell assays were conducted in the absence of metabolic activation. This approach is generally based on a sequential testing system. If a test compound induces a positive response in the absence of metabolic activation, it would be considered a direct-acting chemical. Thus, evaluation of the test compound in the presence of metabolic activation would not be necessary.

The two compounds evaluated, DMMP and JP-10, produced negative responses in the CHO/HGPRT, CHO/SCE and BALB/c-3T3 assays and only a marginally positive response in the CHO/CA assay, indicating that these two compounds are not direct-acting mutagens/carcinogens. Under the circumstances we recommend that these two compounds be retested in assays where validated metabolic activation systems are available (CHO/HGPRT, CHO/SCE and CHO/CA assays). In preliminary studies we found that JP-10 induced a higher frequency of chromosome aberrations in the presence of a microsomal S9 fraction suggesting that this material could be activated to one or more genotoxic agent.

In the Ames assay, JP-10 was tested by a preincubation modification with a 30 minute pretreatment period and was quite toxic even at 1 µl/plate. Shortening the treatment time from 30 to 10 minutes may circumvent the toxicity problem in the Ames assay; therefore, such an experiment which is outside the scope of our current work order is recommended.

TABLE 21

EVALUATION OF DMMP AND JP-10 IN A BATTERY OF
IN VITRO SHORT TERM ASSAYS

SUMMARY

Test Compound / Metabolic Activation	DMMP		JP-10	
	Without	With	Without	With
Ames Assay	-[a]	-	≠[b]	≠
CHO/HGPRT Assay	-	N.D.[d]	-	N.D.
CHO/SCE Assay	-	N.D.	-	N.D.
CHO/CA Assay	?[c]	N.D.	?	N.D.
Balb/c-3T3 Transformation	-	N.D.	-	N.D.

[a] - = Negative response

[b] ≠ = Inconclusive, due to toxicity

[c] ? = Marginally positive response

[d] N.D. = not done

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TECHNICAL REVIEW AND APPROVAL

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This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

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